USE OF THE FLUORESCENT ANTIBODY METHOD IN STUDYING ANTIGEN-ANTIBODY REACTION IN TISSUES. STUDY OF LOCALIZATION OF ANTIRENAL AND ANTIHEPATIC ANTIBODIES IN HOMO- AND HETEROLOGOUS TISSUES

TRANSLATION NO. 1148

August 1964

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

CA 18-064-D4-00019(A) (T-217-1)
10 August 1964

Translation No. 1148

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this document from DDC.

This publication has been translated from the open literature and is available to the general public. Non-DOD agencies may purchase this publication from Clearinghouse for Federal Scientific and Technical Information, U. S. Department of Commerce, Springfield, Va.

Technical Library Branch
Technical Information Division

USE OF THE FLUORESCENT ANTIBODY METHOD IN STUDYING ANTIGEN-ANTIBODY REACTION IN TISSUES. STUDY OF LOCALIZATION OF ANTIRENAL AND ANTIHEPATIC ANTIBODIES IN HOMO- AND HETEROLOGOUS TISSUES

- USSR -

/Following is the translation of an article by I. T. Kolker in the Russian-language publication Khirurgiya i Anesteziologiya (Surgery and Anesthesiology), No 8, March-April, 1963, pages 57-64./

From the Microbiology Laboratory (Director -- Doctor of Medical Sciences I. Ya. Uchitel') of the Institute of Surgery imeni A. V. Vishnevskiy (Director -- Full Member of the Academy of Medical Sciences USSR Professor A. A. Vishnevskiy) of the Academy of Medical Sciences USSR.

It has been found that antiorgan antisera do not exhibit strictly pronounced organ-specificity and enter into reaction not only with homologous, but with other heterologous organs. Organ-specificity is weakly pronounced for certain sera (nephro- and hepatotoxic), while for others (for example, against the cornea and brain tissue) -- to a great extent (A. V. Vishnevskiy; M. M. Kapichinokov; Pressman).

Many investigators have attempted to study the antigenic structure of different organs and tissues. However, the old standby of immunological methods do not permit any conclusions as to where the antigens which give rise to overlapping reactions are located, and to precisely for what tissue components does the reaction antigen-antibody take place. Therefore, in studying these questions we used the visible marker method, that is, the method of fluorescing antibodies.

We attempted, by using labeled antibodies for specific organs, to investigate distribution of active antigenic components in the organs, both analogous and heterologous, and also to study the localization of antibodies, where the antigen- antibody reaction takes place.

In the study, antirenal and antihepatic sera were used, obtained after a two-month immunization of rabbits with a 10 % suspension of homologous organs using the Smadel, somewhat modified by the authors. From the sera (precipitation titers: for antirenal serum -- 1:1280 and antihepatic -- 1:640) a globulin fraction was separated at 50 % ammonium sulfate saturation and combined with fluoresceinisothiocyanate (British preparation) after the method of Riggs et al. The globulin fraction of the sera was added to the reacting mixture (on the basis of 1.5 % protein of the total mixture volume), 20 % carbonate-bicarbonate buffer, and 0.5 M solution with pH 9.0; the mixture was brought to the required volume by adding saline solution.

Fluoresceinisothiocyanate was used on the basis of 5 milligrams per 100 milligrams of protein and the required amount was dissolved in 0.5 ml of acetone in a temperature range of 0 to -3°. The entire fixing process was carried out in a refrigerator at a temperature between zero and -4° employing the MM-2 electromagnetic mixer for 18 hours. The resulting solution underwent dialysis in a cellophane pouch against buffered saline solution with pH 8.6-8.8 prepared fresh daily until fluorescein traces had completely disappeared in the surrounding solution. Dialysis lasted for 8-10 days. In order to remove the "nonspecific color" all the sera obtained in the experiment, including normal rabbit, were treated with acetone powder prepared from rat liver after the method of Coons, Leduc, and Connolly. This method of freeing from unbound coloring agent and "nonspecific color," as our experience and literature data have indicated (Mellors et al; Riggs et al), was the most sparing and economical; it affords reducing additional denaturation and unavoidable losses due to frequent reprecipitation to free from unbound fluorochrome to the minimum. After adsorption the sera were preserved with merthiolate in 1:10,000 dilution ratio stored in a refrigerator at a temperature between zero and four degrees.

The study was carried out on 20 white male rats, weighing 150-200 grams each. The rats were given ether narcosis, they were secured, and their organs were perfused through the vena cava superior with 0.15 M NaCl solution (until total clearance of organ lumens) to free them from blood. Material was taken from the rinsed organs (kidneys, lungs, spleen, liver, and heart) and were frozen in liquid carbon dioxide.

Sections 6-8 microns thick were obtained from the frozen tissues. As a rule, from the organs of each rat 3-5 sections were prepared for staining with antirenal serum, and 3-5 sections for control

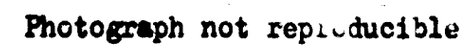
preparations. The sections were stained with fluorescing antibodies after Coons. This study made use of the direct stain method for 45 minutes followed by rinsing of the preparations in buffered saline solution. The sections prepared were mounted in buffered glycerine and examined under the ML-1 luminescence microscope using the 2- SS-4 and 2- SS-8 light filters and the barrier ZhS-18 filter. All the microphotographs were taken by the "Zenith" camera and KF-3 photographic film used with the ML-1 microscope. For comparative study, histological preparations (using a paraffined cutter /provodka/) 3-5 microns thick were prepared from the same organs, which sections were stained with hematoxylin and eosin.

Initially, the kidney sections were treated with antirenal fluorescing globulin. When we studied these sections at small magnitifcation (20 X 4 and 20 X 5) we immediately noted the absence of fluorescence in the medullary substance of the kidney (weak fluorescence of dull-gray color, in intensity only slightly exceeding fluorescence of control preparations) and intensive fluorescing of the cortical layer, in which the renal bodies were distinguished by intensity of fluorescence (Figure 1). When treating sections from the same kidney (control preparations) with normal rabbit serum (combined with fluorochrome according to the scheme described above) only very weak fluorescence of the entire tissue was observed without pronounced differentiation in the various parts of the kidney (Figure 2).

Since the rabbits were immunized with a water-salt extract of all kidney tissues, the antisera obtained must have contained an antibody against the entire organ. Thus, the more intense fluorescence of the cortical layer in comparison with the medullary is evidently due to the principal antigenoactive components of renal tissue being concentrated in the cortical layer, while the medullary renal layer had almost no antigenic properties.

These conclusions agreed with the data of Heymann and Lund, who established that nephrotoxic serum, obtained from cortical tissue, is significantly more active than serum obtained from the medullary substance of the same kidney. This also is pointed out in the studies of Pressman et al, who found by using the radioactive marker method that I^{131}- labeled antirenal serum accumulates chiefly in the cortical layer.

Study of kidney sections at high magnification is of definite interest, allowing us to discover distribution of antigenoactive substances in the renal glomeruli and tubules. The most intensive fluorescence was noted in renal glomeruli and in the basal membrane of renal tubules, while all the remaining tissue glowed dark-gray. Study of individual renal glomeruli at high magnification (40 X 10 and 40 X 15) shows that these glomeruli themselves are also nonuniform in the antigenic sense. The Shumlyanskiy capsule stands apart in the renal



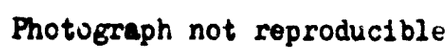


Figure 1. Section of kidney treated with antirenal fluorescing globulin. Magnification: 100 X.

Figure 2. Section of kidney treated with normal labeled rabbit globulin. Magnification 200 X.

body by its intensity of fluorescence, as if the glomerulus itself had been enclosed by a fluorescing layer; the fluorescing individual capillaries of the glomerulus were distinctly visible (Figure 3, a).

Figure 3. Sections of kidney treated with antirenal fluorescing globulin. a - renal glomerulus. Magnified 400 X; b - renal tubules. Magnified 600 X.

When we examined individual renal tubules at a magnification of 40 X 15 (fixed sections obtained by using paraffin cutter /provodka/ we noted fluorescence of the basal membrane, appearing to envelop this tubule, and fluorescence of the epithelial cytoplasm of the tubules, while the nuclei of the cells did not fluoresce, which is evidently due to their lack of substances antigenic to the staining antibody (Figure 3, b).

In order to demonstrate that differences in fluorescence intensity of tissue components is a consequence of specific immunohistochemical reaction between the tissue antigen and the labeled antibody, we made the appropriate control investigations.

1. Suppression of specific fluorescence by unstained antibodies.

Photographs not reproducible

Figure 4. Kidney section treated with antirenal unlabeled globulin and additionally by antirenal fluorescing globulin. Magnified 200 X.

Figure 5. Kidney section treated with normal rabbit globulin and additionally with antirenal fluorescing globulin. Magnified 200 X.

For this purpose, two-three drops of antirenal unlabeled globulin were placed on the kidney sections and the preparations were treated according to the scheme described (additional storage in refrigerator at 4° for two hours). After this, the sections were carefully rinsed four-five times over 30 minutes in fresh portions of buffered saline solution and then were treated with antirenal fluorescing globulin under the usual scheme. A sharp decrease in fluorescence of the preparations was noted, along with the lack of any fluorescence in the basal membrane of the renal tubules and almost total suppression of fluorescence in the renal glomeruli (Figure 4).

During this study, we were convinced that still more complete suppression of fluorescence is attained in those cases when the whole homologous antiserum is used for "exhaustion," and not its globulin fraction. Thus, in processing the kidney sections with initially unlabeled antirenal whole serum (for "exhaustion"), and then with labeled antirenal globulin, we observed total suppression of fluorescence (only weak background fluorescence was observed).

In order to prove that suppression of specific fluorescence in the previous control sample is specific and due to lack of free antigen (which reacts with unlabeled antibody), we treated beforehand analogous kidney sections with unlabeled normal rabbit globulin and additionally with antirenal fluorescing globulin according to the scheme described (Figure 5). In this case, we observed no suppression of intensity of preparation fluorescence; intensive fluorescence was noted in the renal glomeruli and in the basal membrane of the tubules. The fact that the preliminary treatment of the sections with serum containing antibodies to the particular organ impeded localization of labeled antibodies (accordingly, preparation fluorescence also decreased, while treatment of sections with normal rabbit globulin barely at all reduced fluorescence intensity, allowed us to conclude that the fluorescence observed is due to the specific immunological reaction between tissue antigen and labeled antibody.

2. Control for "exhaustion" of antiserum.

Photographs not reproducible

Figure 6. Sections of rat (a) lung, (b), spleen, and (c) liver, treated with antirenal fluorescing globulin. Magnification: 200 X.

In order to show that the fluorescence observed is caused by specific seral antibodies, we conducted several control experiments on the "exhaustion" of the labeled sera. In the test tube, 1 ml of antirenal labeled serum was mixed with the precipitate obtained from 2-3 mls of antirenal suspension. The mixture was carefully shaken and stored in a thermostat at a temperature of

37° for 2-3 hours, and then left overnight in a refrigerator at 4°. On the next day, the mixture was centrifuged at 3500 rpm for 15-20 minutes and the precipitate was rejected, while the supernatant liquid was used for staining. In the staining with this adsorbed section serum, a sharp decrease in fluorescence intensity compared to experimental samples was observed and the outlines of the renal body were barely noticeable in the microphotographs.

3. When the kidney sections were treated with normal rabbit serum previously adsorbed with a mixture of acetone powders from all rat organs, no specific indications as to location of antigens was found and only general weak fluorescence of the entire tissue was observed (cf Figure 2).

Thus, comparison of the control preparations with experimental showed that the fluorescence described is the result of specific immunohistochemical reactions and that antirenal antibodies exhibit specific localization in renal corpuscles and in the basal membrane of renal tubules.

Photograph not reproducible

Figure 7. Kidney section treated with antihepatic fluorescing globulin. Magnification: 200 X.

Photograph not reproducible

Figure 8. Liver section treated with antihepatic labeled globulin adsorbed with powder from human liver. Magnification: 200 X.

In order to reveal localization of antirenal antibodies in heterologous rat organs, we treated tissue sections of rat lung, spleen, liver, and heart with antirenal fluorescing globulin according to the method described. Thereupon, we noted immediately that the intensity of fluorescence in preparations made from these organs was considerably lower than fluorescence of kidney sections, which confirmed the organ specificity of the serum studied. Intensity of

fluorescence increases at the sites of blood vessels and capillaries. Intensive fluorescence of epithelium, lining bronchi and alveoli was noted in sections of lung tissue, while the interalveolar interstices did not fluoresce (Figure 6, a); the central artery and blood capillaries along the periphery of a Malpighean corpuscle (Figure 6, b) was easily distinguished in the spleen sections by fluorescence intensity. Fluorescence of sections of liver and heart tissue (Figure 6, c) was slight.

In the second half of the experiment we studied the distribution of antihepatic antibodies in homo- and heterologous organs. It was found that treatment of antihepatic serum with acetone powder from rat liver sharply reduced the intensity of its fluorescence; evidently, partial exhaustion of the serum takes place, therefore in subsequent experimentation we treated the antihepatic serum with human liver acetone powder to eliminate the "nonspecific fluorescence." Upon such a serum treatment of the liver sections, fluorescence of the content of the hepatic channels [balki] leading from the central vein was observed, while the cell nuclei did not fluoresce (Figure 8).

The fluorescence described is specific, since when the appropriate control experiments were carried out for "exhaustion" fluorescence was almost totally suppressed (Figure 9). Subsequently, antihepatic serum adsorbed with human liver acetone powder was used to treat heterologous organs. In so doing, we observed intensive fluorescing of the hepatic glomeruli, and on preparations made from liver tissue -- moderately intense fluorescence of epithelium lining the alveoli, while interalveolar interstices did not fluoresce. Fluorescence was weak on preparations made from spleen and heart tissue.

Photograph not reproducible

Figure 9. Liver section treated with antihepatic unlabeled serum and additionally with antirenal labeled globulin. Magnification: 200 X.

Thus, antirenal antibodies exhibit strongly pronounced specificity as to localization and are located chiefly in the kidney, in particular in the renal corpuscles and in the basal membrane of renal tubules. In addition, antirenal antibodies are localized, but in significantly lesser amounts, in the heterologous organs as well -- in the lungs, spleen, liver, and heart. In contrast /to the localization of antirenal antibodies/, antihepatic antibodies exhibit no selective localization in rat organs and emitted more or less uniform fluorescence in all organs. It is, however, characteristic that in renal tissue they are localized chiefly to the same extent as in the homologous liver.

The data evidenced nonuniformity of renal tissue in the antigenic aspect. Hill and Cruickshank, studying antigenic properties of rat connective tissue, noted the existence in antirenal serum of two antibody groups: some reacted with the basal membrane of renal glomeruli and tubules and the reticular tissue of vessels, and others -- with the epithelium of urinary tubules. The authors also pointed out that it is precisely due to the first antibody group that antirenal serum reacts with other organs, while the antigen present in epithelium of renal tubules is not found in other organs.

Of late, the complex structure of renal tissue has also been evidenced in the studies of Yagi et al, who have shown that there are at least two antigens, and perhaps more, responsible for localization of antirenal antibodies.

Conclusions

1. Antirenal antibodies exhibit selective localization in a homologous organ and are located in lesser amounts in heterologous organs.

2. The maximum in localization of antirenal antibodies has been noted in renal glomeruli, especially in capillary bundles of glomeruli.

3. In heterologous organs antibodies are generally localized about blood vessels and capillaries.

4. Antihepatic antibodies do not manifest this selective localization, distributing more or less uniformly throughout all organs.

LITERATURE

Vishnevskiy, A. V., Russkiy vrach (Russian Physician), 1907, Vol 6, No 42, page 1448.

Kapichnikov, M. M., in the book: Voprosy immunologii normal'nykh i zlokachestvennykh tkaney (Problems of Immunology of Normal and Malignant Tissues), Moscow, 1956, page 177.

Coons, A. H., Leduc, E. H., Connolly, I. M., Journal of Experimental Medicine, 1955, Vol 102, page 49.
Heymann, W., Lund, H., Science, 1948, Vol 108, page 448.
Hill, A. G. S., Cruickshank, R., British Journal of Experimental Pathology, 1953, Vol 34, page 27.
Mellors, R. C., Siegel, M., Pressman, D., Laboratory Investigations, 1955, Vol 4, page 69.
Pressman, D., Annals of the New York Academy of Sciences, 1955, Vol 59, Article 2, pages 227; 376.
Pressman, D., in the book: Nature and Significance of the Antibody Response, New York, 1953, page 207.
Riggs, J. L., Seiwald, R. J., Burckhalter, I. H., et al, American Journal of Pathology, 1958, Vol 34, page 1081.
Smadel, J. E., Journal of Experimental Medicine, 1936, Vol 64, page 921.
Yagi, Y., Pressman, D., Journal of Immunology, 1958, Vol 81, page 7.

- END -